Research article

Biomedical Chromatography

Received 27 September 2010, Revised 18 November 2010, Accepted 19 November 2010 Published online in Wiley Online Library: 4 March 2011

(wileyonlinelibrary.com) DOI 10.1002/bmc.1595

Rapid analysis of lignans from leaves of *Podophyllum peltatum* L. samples using UPLC-UV-MS

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ABSTRACT: A new rapid UPLC-UV-MS method has been developed that permits the analysis of four lignans (4′-*O*-demethylpodophyllotoxin, podophyllotoxin, *α*-peltatin and *β*-peltatin) in *P. peltatum* L. Podophyllotoxin is a natural lignan that is being used as a precursor for the semi-synthetic anti-cancer drugs etoposide, teniposide and etopophos. The chromatographic separation was achieved using a reversed-phase C_{18} column with a mobile phase of water and acetonitrile, both containing 0.05% formic acid. Analyses of *P. peltatum* leaves collected from different colonies within a single site indicated a significant variation in 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin content. Within 3.0 min four main lignans could be separated with detection limits of 0.1, 0.3, 0.3 and 0.2 µg/mL, respectively. 4′-*O*-demethylpodophyllotoxin and *α*-peltatin appeared most prominently among the lignans obtained. The podophyllotoxin content was found in the range of 0.004–0.77% from 16 samples collected from 6 colonies within the same site. The content of podophyllotoxin is directly proportional to the content of 4′-*O*-demethylpodophyllotoxin and inversely proportional to *α*-peltatin and *β*-peltatin content. LC-mass spectrometry coupled with electrospray ionization (ESI) interface method is described for the identification of four lignans in various populations of plant samples. By applying principal component analysis and hierarchical cluster analysis, *Podophyllum* samples collected from various colonies within a location were distinguished. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: *Podophyllum peltatum* L.; podophyllotoxin; lignans; UPLC-UV-MS

Introduction

The American mayapple (*Podophyllum peltatum* L.) is a rhizomatous perennial species found throughout the wooded landscape of the eastern half of North America. The potential for the development of *P. peltatum* as a cash crop for rural America was suggested 35 years ago and re-visited in 1998 (Meijer, 1974; Moraes *et al*., 2000). Etoposide, teniposide and etopophos, three drugs used against testicular and small-cell lung cancer, are semisynthetic products derived from podophyllotoxin, a lignan occuring in different *Podophyllum* species (Podophyllaceae/Berberidaceae; Ganzera *et al*., 1999). The successful derivatization of podophyllotoxin into these potent antineoplastic drugs has generated interest in structure optimization in order to produce new derivatives with superior pharmacological profiles and broader therapeutic uses. The administration of podophyllotoxin and 4′-*O*-demethylpodophyllotoxin-derived drugs causes complex physiological reactions beyond inhibition of DNA topoisomerase and tubulin polymerization (Moraes *et al*., 2002). Podophyllotoxin is also a precursor to a new derivative, CPH 82, that is being tested for rheumatoid arthritis in Europe, and it is the precursor to other derivatives used for the treatment of psoriasis and malaria (Moraes *et al*., 2002). Several podophyllotoxin preparations are on the market for dermatological use to treat genital warts (Moraes *et al*., 2002). Since the total synthesis of podophyllotoxin is an expensive process, availability of the compound from natural resources is an important issue for pharmaceutical companies that manufacture these drugs (Moraes *et al*., 2002). So far, the commercial source of this compound has been *P. emodi*, natively found in India, but due to over-exploitation, this species has been declared endangered. The American species *P. peltatum* might be considered as an alternative commercially viable source (Moraes *et al*., 2000) for podophyllotoxin production. In contrast, *P. peltatum* stores podophyllotoxin 4-*O*-*β*-D-glucopyranoside in leaf blades, and recent developments have shown that this compound can easily be converted to podophyllotoxin (Moraes *et al*., 2002; Canel *et al*., 2001). The findings suggest that *P. peltatum* is a better candidate for development as a sustainable crop than *P. emodi* because, as a perennial herb, flowering in May, it generates leaves annually from the rhizome. Leaves can be harvested late in the season without damaging the plant (Cushman *et al*., 2006). In addition to the above-mentioned pharmaceutical properties, podophyllotoxin, along with *α*-peltatin and *β*-peltatin, are aryltetralin lignans known to have other associated biological activities, such as anti-cancer, anti-fungal,

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anti-viral, anti-mitotic and immunostimulatory properties (Rahman *et al*., 1995; Goel *et al*., 1998; Pugh *et al*., 2001).

An API-LC/MS/MS method was developed for the identification of *P. emodi* based on the profile of its aryltetrahydronaphthalene and related lignan marker compounds (Wong *et al*., 2000). Ganzera *et al*. (1999) reported the separation of *Podophyllum* lignans by micellar electrokinetic capillary chromatography (MECC). An HPLC method for the simultaneous separation and determination of *Podophyllum* lignans in *Podophyllum* species was established by Liu and Jiao (2006) and Bastos *et al*. (1995). Liu and Jiao (2006) determined podophyllum lignans and flavonoids in *P. emodi* Wall. var. *chinensis* Spargue. Bastos *et al*. (1996) reported the determination of podophyllotoxin and related compounds in *Podophyllum* species. Analytical methods described in the literature are based on TLC, CE (Ganzera *et al*., 1999), API-LC/MS/MS (Wong *et al*., 2000), HPLC (Liu and Jiao, 2006; Bastos *et al*., 1995, 1996) either for the determination of lignans or for podophyllotoxin alone. In the present work, a new highly sensitive and rapid UPLC-UV-MS method was developed to analyze four lignans [4′-*O*-demethylpodophyllotoxin (**1**), *α*-peltatin (**2**), podophyllotoxin (**3**) and *β*-peltatin (**4**), Fig. 1] from leaves of *P. peltatum*. Podophyllotoxin is an important compound for the pharmaceutical industry, and screening of large numbers of samples from different samples to discover higher yielding sources is important. In order to secure a podophyllotoxin supply and to evaluate the chemo-diversity of *P. peltatum* within a population, 16 leaf samples were harvested from six distant colonies within the same location. Colony I provided five samples coded as H-1 to H-5. Colony II provided two samples coded H-6 and H-7. Samples H-8 and H-9 were collected from colony III; H-10 and H-11 were from colony IV while colony V produced samples H-12 and H-13 and colony VI provide H-14 to H-16. Colonies I and V were 50 m apart. Sample distance within the colony was approximately 1 m apart while, the distance between colonies varied from 5 to 50 m apart. The newly developed UPLC method for quantitative determination of lignans was found to be capable of giving shorter retention times while maintaining good resolution and sensitivity.

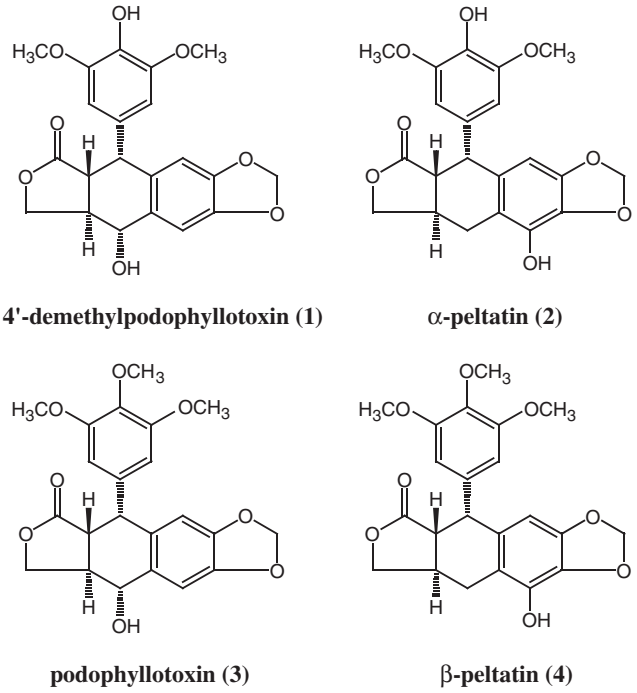

Figure 1. Structures of lignans (**1–4**).

Experimental

Instrumentation and chromatographic conditions

Ultra-performance liquid chromatography–UV–MS. All analyses were performed on a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) including binary solvent manager, sampler manager, column compartment and PDA (Waters Acquity model code UPD) connected to Waters Empower 2 data station. An Acquity UPLC™ BEH Shield RP_{18} column (50 × 2.1 mm i.d., 1.7 µm), also from Waters, was used. The column and sample temperature were maintained at 40 and 25°C, respectively. The column was equipped with an LC-18 guard column (Vanguard 2.1 × 5 mm, Waters Corp., Milford, MA, USA). The mobile phase consisted of water (A), and acetonitrile (B) both containing 0.05% formic acid at a flow rate of 0.25 mL/min with an isocratic elution as follows: 0 min, 68% A–32% B held for 3 min. Each run was followed by a 2 min wash with 100% B and an equilibration period of 3 min with 68% A–32% B. Strong needle wash solution (95:5, acetonitrile–water) and weak needle wash solution (10:90, acetonitrile–water) were used. All of the solutions mentioned were filtered via 0.20 µm membranes under vacuum and degassed before their usage. The total run time for analysis was 3 min and detection wavelength at 210 nm. Two microliters of sample was injected and peaks were assigned with respect to the mass of the compounds and comparison of the retention times.

The effluent from the LC column was directed into the ESI probe (Acquity UPLC-SQD). Mass spectrometer conditions were optimized to obtain maximal sensitivity. The source temperature and the desolvation temperature were maintained at 150 and 350°C, respectively. The probe voltage (capillary voltage), cone voltage and extractor voltage were fixed at 3.0 kV, 60 V and 3 V, respectively. Nitrogen was used as the source of desolvation gas (650 L/h) and drying gas (25 L/h). Analytes were identified in selected ion monitoring (SIM) mode. $[M + Na]^+$ = 423.1 (compounds **1** and **2**) and 437.1 ions for compounds **3** and **4** were selected as detecting ions. Mass spectra were obtained at a dwell time of 0.5 s in SIM and 500 Da/s of scan rate. A mass range of 150–900 amu was scanned.

Chemicals. The standard compounds (**1–4**) were isolated at NCNPR, and their identity and purity were confirmed by chromatographic (TLC, HPLC) methods, by the analysis of the spectral data (IR, 1D- and 2D-NMR, HR-ESI-MS) and comparison with published spectral data (Bastos *et al*., 1996; Moraes *et al*., 2005; Jackson and Dewick, 1984). Acetonitrile, water and formic acid were of HPLC grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Standard solutions for accuracy and precision determination. An individual stock solution of standard compounds was prepared at a concentration of 0.1 mg/mL in methanol. The calibration curves were prepared at five different concentration levels, in triplicate, once each day and were used to assess accuracy and precision of the assay method. The range of the calibration curves was from 0.8 to 100 µg/mL by the LC-UV method. Table 1 shows the LC-UV calibration data and the calculated limits of detection for each method.

Sample preparation

The extraction of lignan content was conducted according to the method described by Canel *et al*. (2001), and the resulting residue was dissolved in 10.0 mL methanol. The final extract was filtered through a syringe filter (0.2 µm) prior to analysis. UPLC-UV-MS analysis, the more sensitive method, required dilutions of 2–10 times. The injection volume was 2 µL.

Plant samples. Leaf samples of 16 mayapple accessions were collected from six colonies from a single site located at Lafayette county MS already reported in our previous work as LAMS 1 and LAMS 2 and described as shade colonies. The mayapple colonies in this study were

Table 1. Regression equation, correlation coefficient (r^2), limit of detection (LOD) and limit of quantitation (LOQ) for four lignans using UPLC-UV method at 210 nm

Analyte	Regression Equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
1	$y = 3.36 \times 10^4 x - 4.86 \times 10^3$	0.9997	0.1	0.5
2	$y = 1.87 \times 10^4 x + 1.39 \times 10^4$	0.9981	0.3	0.8
3	$y = 1.53 \times 10^4 x + 9.31 \times 10^3$	0.9973	0.3	0.8
4	$y = 2.68 \times 10^4 x + 8.98 \times 10^3$	0.9991	0.2	0.8

distributed in an area of approximately 1.2 ha. Within this area, six distant colonies varying in sizes were harvested. Specimens of this population were pressed and sent to the Pullen Herbarium, Department to Biology at the University of Mississippi.

Results and discussion

Chromatographic conditions

In the UPLC-UV analysis, various mobile phase compositions and chromatographic conditions were tested to find the optimal chromatographic conditions. A mobile phase containing water and acetonitrile, both containing 0.05% formic acid, and a constant flow rate at 0.25 mL/min on an Acquity UPLC™ BEH shield RP_{18} (50 × 2.1 mm, 1.7 μm i.d.) column using isocratic elution and a fixed column temperature at 40°C were found to be the optimal separation conditions for determination of compounds **1–4** in samples. Baseline resolution was obtained and good peak shapes observed without tailing. The different columns tested were Acquity UPLC™ Bridged Ethyl-siloxane/silica Hybrid (BEH) C_{18} (100 × 2.1 mm i.d., 1.7 μm), Acquity UPLC™ BEH C_{18} (50 × 2.1 mm i.d., 1.7 μm) and Acquity UPLC™ BEH Shield RP_{18} (embedded polar group column). Each provided a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes. Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C_{18} column; an acidic system improved the sensitivity and proton enhancement. Variation of the column temperature between 25 and 40°C did not cause significant change in the resolution, although changes in retention time were observed.

Comparison study of HPLC and UPLC performance

The analytical conditions for the tested columns were chosen considering the method to be frequently used in routine analysis while maintaining the speed, sensitivity and resolution of analysis. The chromatographic conditions originally developed for *Podophyllum* analysis are described in previous papers (Bastos *et al.*, 1995, 1996). Under these conditions all four compounds **1–4** of *Podophyllum* were well separated, meeting the criteria of validation requirements. Originally, the analytical run took about 25–35 min (Bastos *et al.*, 1995, 1996). It is a long run time for a series of routine analyses. Therefore, modern developments in LC were applied in order to save time and solvent consumption. The sensitivity of UPLC system was good even for such low injection volumes as 2 μL. The limits of detection with previous reported methods were between 2.5 and 5 μg/mL (Bastos *et al.*, 1995, 1996), and with the UPLC system were in the range of 0.1–0.3 μg/mL. As UPLC functions according to the chromatographic principles and separation mechanism of HPLC, method transfer and revalidation were quite easy and especially time saving.

Accuracy, precision and linearity. The five-point calibration curves for the four lignans showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity ($r^2 > 0.99$) of the detector response for all standard compounds from 100 to 0.8 μg/mL. The limits of detection and limits of quantification for the four lignans (**1–4**) were found to be in the range of 0.1–0.3 and 0.5–0.8 μg/mL, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were defined,

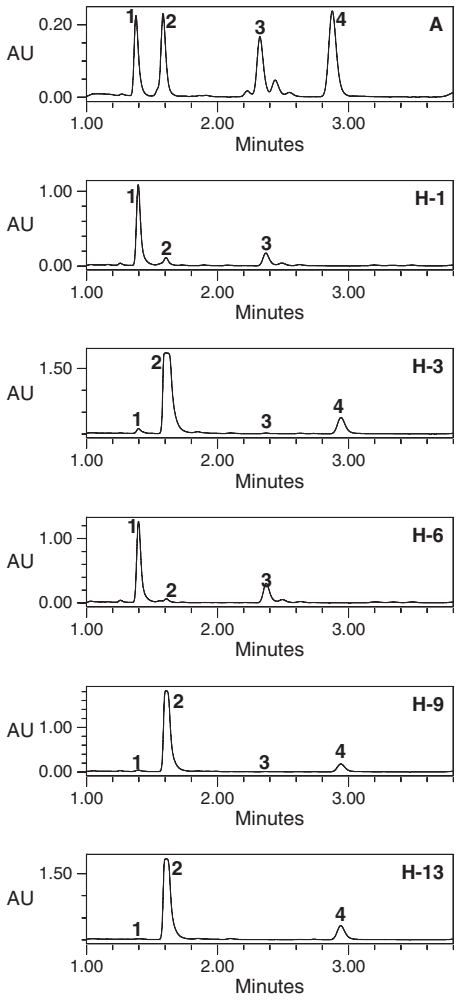

Figure 2. UPLC-UV chromatograms of standard mix (A) and plant samples (H-1, H-3, H-6, H-9 and H-13) using a PDA detector at 210 nm: (**1**) 4′-*O*-demethylpodophyllotoxin, (**2**) *α*-peltatin, (**3**) podophyllotoxin and (**4**) *β*-peltatin.

respectively, as signal-to-noise ratio equal to 2 or 3 and 10. Accuracy of the method was confirmed by performing a recovery experiment. Samples of *Podophyllum* were spiked with known amounts of the standard compound, extracted and analyzed. All standards and samples were injected in triplicate. Multiple injections showed that the results were highly reproducible with low standard error. Intra- and inter-day variation of the assay was determined to be lower than 4.0%, with a maximum RSD of 3.73. This was performed three times on three different days and each concentration was injected in triplicate.

Analysis of plant samples. Figure 2 shows the LC-UV chromatograms for the standard compounds and *P. peltatum* samples. Figure 3 shows the LC-MS chromatograms of lignans in various plant samples. The method was applied for the quantification and qualitative analysis of four lignans in different populations of *P. peltatum*. Overall, the concentrations of compounds **1–4** in the collected samples ranged from not detectable to detectable levels to 7, 36, 8 and 3 mg/g dry weight, respectively (Table 2). The content of 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin were in the ranges 0.007–0.70, 0.033–3.59, 0.004–0.77 and 0.005-0.31%, respectively (Table 2). The highest contents of 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin were present in samples H-5 (0.7%) (**1**), H-3 (3.59%) (**2**), H-5 (0.77%) (**3**) and H-14 (0.31%) (**4**), respectively. The lowest contents of the four lignans were present in samples H-12 to H-14 (0.007%) for 4′-*O*-demethylpodophyllotoxin, H-6 (0.033%) for *α*-peltatin, H-9 (0.004%) for podophyllotoxin and H-4 (0.005%) for *β*-peltatin. All 16 samples showed the presence of the two lignans (compounds **1** and **2**) analyzed. Samples H-10 to H-16 did not show for the presence of compound **3** but showed high content of compound **2**. Samples H-1, H-2 and H-5 to H-7, showed high contents of compound **1** but compound **4** could not be detected. Figure 4 shows the variation in the content of lignans in *P. peltatum* leaves collected on 28 May 2010 in the site located at

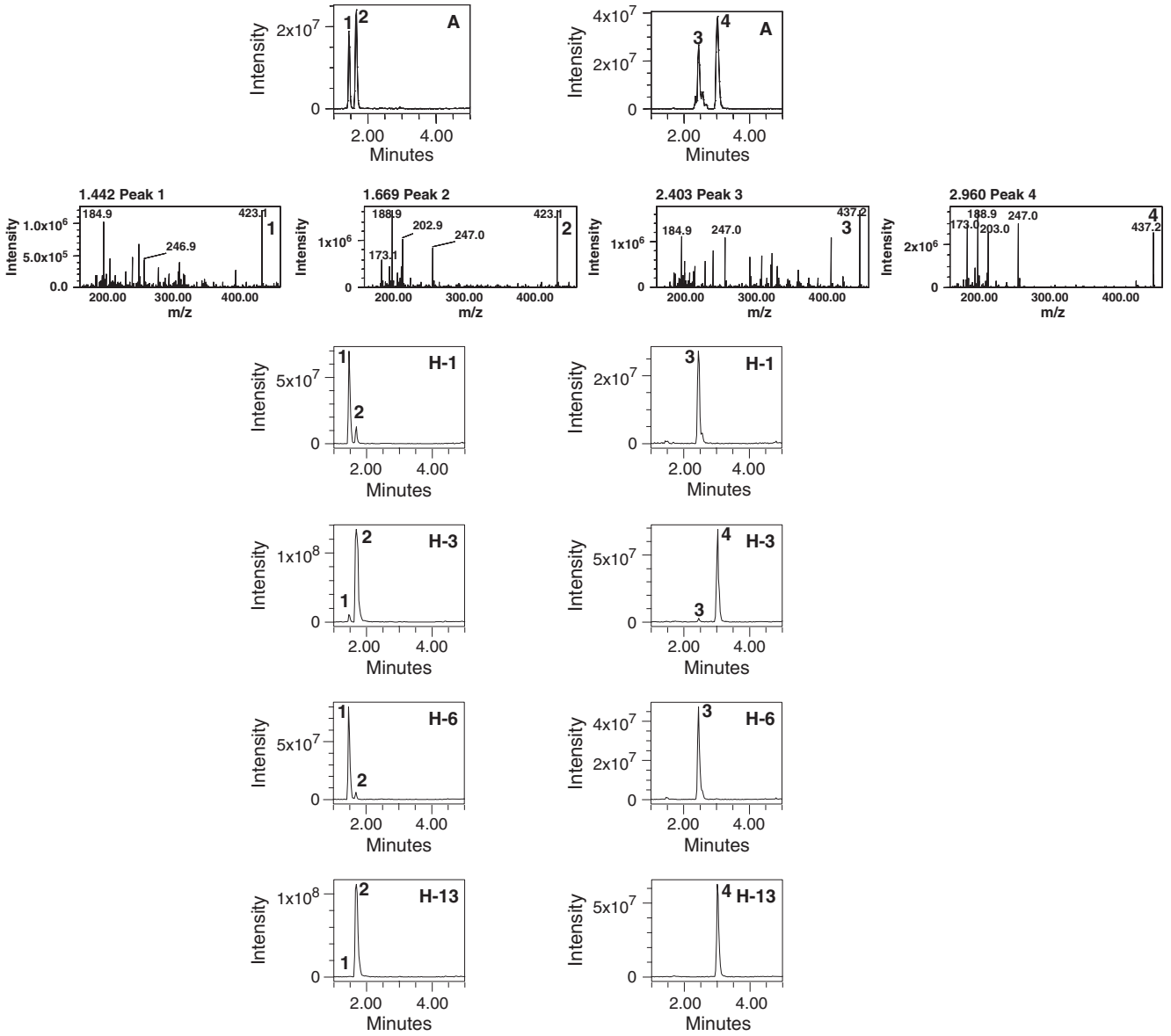

Figure 3. Single ion chromatograms of standard mix-4 (A) and various populations of *P. peltatum* (H-1, H-3, H-6 and H-13).

Table 2. The contents (%, mg/100 mg dry weight) of lignans in *P. peltatum* leaves

Sample	1	2	3	4
H-1	0.303	0.063	0.146	ND
H-2	0.698	0.137	0.395	ND
H-3	0.034	3.595	0.007	0.233
H-4	0.654	0.234	0.437	0.005
H-5	0.703	0.143	0.771	ND
H-6	0.388	0.033	0.252	ND
H-7	0.681	0.081	0.416	ND
H-8	0.025	1.955	0.008	0.100
H-9	0.013	2.132	0.004	0.111
H-10	0.017	2.687	ND	0.090
H-11	0.025	3.876	ND	0.214
H-12	0.007	2.309	ND	0.105
H-13	0.007	2.552	ND	0.205
H-14	0.007	3.290	ND	0.311
H-15	0.010	2.528	ND	0.154
H-16	0.010	2.127	ND	0.170

ND, not detected.

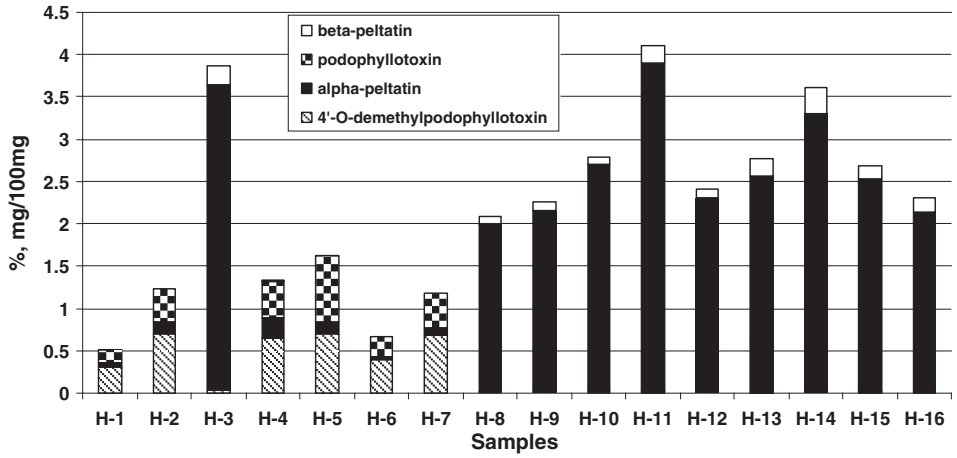

Figure 4. Variation of 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin in *P. peltatum* colonies collected from a single population located in Oxford, MS, USA.

Old Taylor Road at the University of Mississippi Campus. There was a significant variation in the 4′-*O*-demethylpodophyllotoxin, podophyllotoxin, *α*-peltatin and *β*-peltatin contents, which demonstrated that there is significant chemo-diversity within a single population. Previously, we have reported two accessions as being LAMS 1, a podophyllotoxin rich accession producing 3.46%, and LAMS 2, peltatin-rich with contents of 1.65% *α*-peltatin and 1.38% *β*-peltatin (Moraes *et al*., 2005).

LC-MS is a powerful qualitative and quantitative technique for the determination of molecular masses of analytes, because analyte identification on the basis of molecular mass is extremely selective. This method involved the use of the $[M+Na]^+$ ions of 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin at *m/z* 423.1, 423.1, 437.1 and 437.1, in the positive ion mode with SIM. With this method, compounds **1–4** were detected in ppb levels. No interfering peaks were found at the retention time of interest. Further, the fragmentation patterns observed in the mass spectrum were useful in characterization of the compounds. The key fragments of compounds **1–4** were separated as two groups for compounds **1** and **3**, and compounds **2** and **4**, respectively. Within each group, compounds shared very similar fragmentation patterns. Fragments of 4′-demethylpodophyllotoxin and podophyllotoxin were *m/z* 247 $[M-C_8H_9O_3]^+$ or $[M-C_9H_{11}O_3]^+$ and 185 $[247-C_2H_6O_2]^+$ of *α*-peltatin and of *β*-peltatin *m/z* 247 $[M-C_8H_9O_3]^+$ or $[M-C_9H_{11}O_3]^+$, 203 $[247-CO_2]^+$, 189 $[203-CH_2]^+$ and 173 $[203-O]^+$. The elimination of a trimethoxybenzene molecule produced the *m/z* 247 ion. The signal of the *m/z* 247 ion was intense, possibly because of its high stability (Fig. 3).

Principal component analysis and hierarchical cluster analysis

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to 16 samples on the chemical parameters in order to investigate and visualize the homogeneity

of the samples (Figs 5 and 6). HCA and PCA were performed using the XLSTAT version 2010.4.03 add-in for Microsoft Excel and Pirouette (version 4.0) Infometrix Inc. (Bothell, WA, USA). Although it is possible to visually differentiate the different chromatograms, the process is subjective and not quantitative. In addition, minor differences between very similar chromatograms might be missed.

The principal components (PC) can be displayed graphically as a scores plot. This plot is useful for observing any groupings in the data set. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Massart *et al*., 1988). Generally, this separation took place in the first two principal components (PC-1 and PC-2). Examination of the scores and loading plots for PC-1 vs PC-2 showed that compounds **1** and **3** are clearly separated from the compounds **2** and **4**. The separation is due mainly to PC-1. The difference obtained by PC-2 has no effect on the separation of compounds because the same samples show a less reproducible value when compared with the PC-1 value. The samples H-3, H-11 and H-14, with high lignan contents, were distinguished from other samples (H-2, H-4 to H-7). Samples H-2, H-4 and H-7 were closely related and some of the other samples (H-5) were positioned apart from sample H-14 due to specific patterns of compounds **2** and **3**. Samples H-1 and H-6, with low lignan contents, are positioned away from the high lignan content samples (H-2, H-4 and H-7). Samples H-8 to H-10, H-12, H-15 and H-16 were closely related and grouped away from sample H-5 due to high content of compound **2**. The contributions of the first and second principal component obtained, using the peak area data of compounds **1–4**, were together 97.06%, representing variation in the data.

The present PCA results obtained were similar to those obtained by hierarchical cluster analysis. Indeed, homogeneous samples originating from the same small scale facility were gathered in the same cluster. The results of HCA are shown in Fig. 6. It was clear that the samples could be divided into three clusters: samples H-10, H-13, H-15, H-8, H-12, H-9 and H-16 in cluster one; samples H-3, H-11 and H-14 in cluster two; and samples H-7, H-2, H-4, H-6, H-1 and H-5 in cluster three. This result agreed very well with visual comparisons of their chromatograms.

In conclusions, the newly developed UPLC method for four lignans was found to be capable of giving shorter retention times while maintaining good resolution as compared with conventional HPLC. This method exhibited an excellent performance in terms of sensitivity and speed. It is a suitable method for rapid analysis of lignans and for chemical fingerprint analysis. The developed method was validated for all the parameters tested and successfully applied to the identification in various populations of *P. peltatum*. The UPLC profiles of various plant samples were compared and showed variations in the lignan content. Cluster and principal component analysis for podophyllotoxin indicated that mayapple colonies in close proximity may exhibit significant differences as clusters with respect to 4′-*O*-demethylpodophyllotoxin, *α*-peltatin, podophyllotoxin and *β*-peltatin. Based on the statistical or data analysis, mayapple could be divided into four major groups with respect to podophyllotoxin concentration, with H-5 (0.8%) as group 1; H-7, H-2 and H-4 as group 2 (0.4%); H-6 and H-1 as group 3 (0.2%); and H-11, H-14, H-10, H-13, H-15, H-12 and H-16 as group 4, where podophyllotoxin was not detected. The results from this study suggest that there is great variation in lignan concentrations in mayapple samples where there are chemotypes within a population and neighboring accessions may not necessarily belong to the same colony and the same chemotype group. The new UPLC technique led to a reduction in the mobile phase flow rate; other effects were an increase in acquisition rate and decrease in injection volumes to achieve good peak shapes. The time required for method development, column equilibration or re-equilibration while using gradient elution and for method

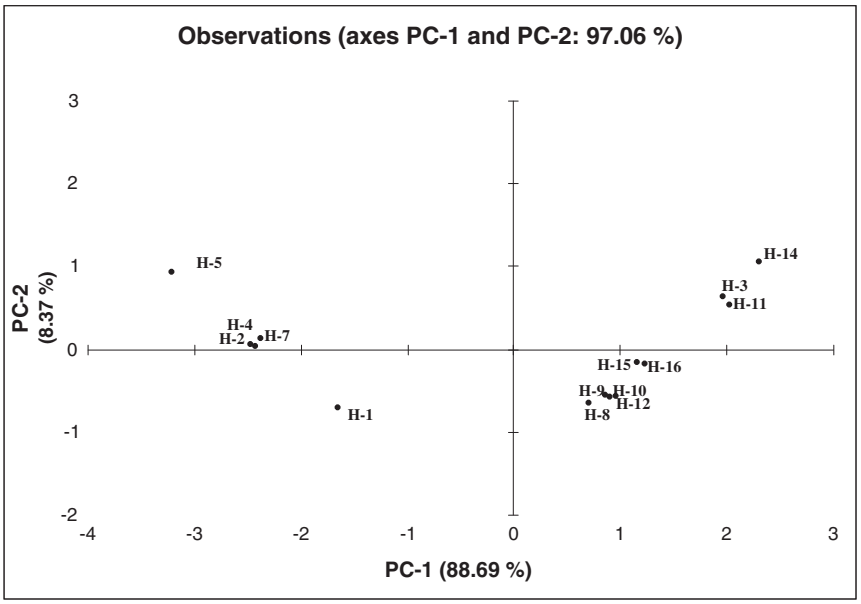

Figure 5. *Podophyllum* samples; scatterplot of first and second principal component obtained by PCA on the basis of UPLC peak area data of (**1**) 4′-*O*-demethylpodophyllotoxin, (**2**) *α*-peltatin, (**3**) podophyllotoxin, (**4**) *β*-peltatin; first principal component: eigenvalue 3.559, contribution 88.97%; second principal component: eigenvalue 0.321, contribution 8.034%. H-13 was excluded from PCA.

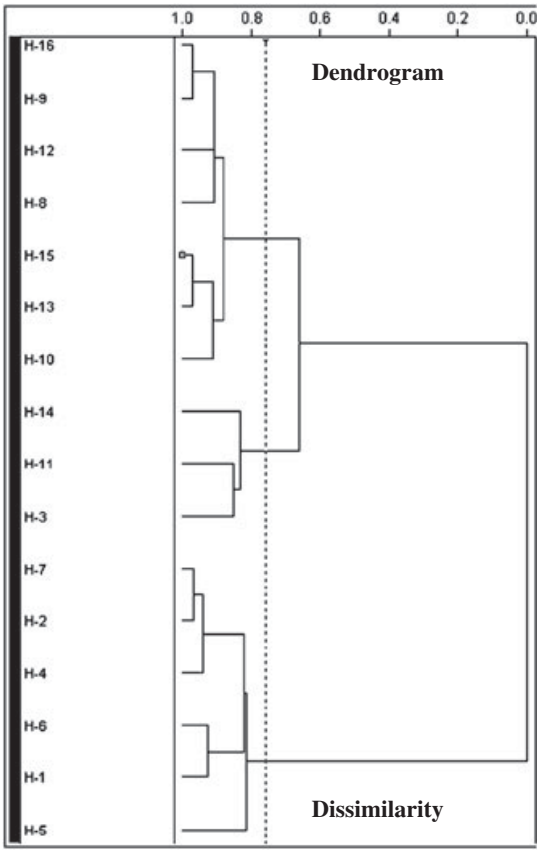

Figure 6. Dendrogram derived from the HCA performed on the chemical data obtained from the measurements in different locations. The dashed line shows the level of statistical significance of the discrimination.

validation was much shorter compared with conventional HPLC system. This study was conducted to investigate the chemo-diversity present at the same location.

Acknowledgments

This research was funded in part by 'Science Based Authentication of Dietary Supplements' and 'Botanical Dietary Supplement Research' funded by the Food and Drug Administration grant numbers 5U01FD002071-09 and 1U01FD003871-01, and the United States Department of Agriculture, Agricultural Research Service, Specific Cooperative Agreement no. 58-6408-2-0009.

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